FINAL REPORT

CONTRACT NO. DA-18-064-404-CML-495

DEVELOPMENT OF FLUORESCENT COMPOUNDS AND REDOX INDICATOR DYES

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SECTION I

SCOPE OF THE INVESTIGATION

A. General Nature of the Problem.

The present report is a summary of the investigation under Contract No. DA-18-064-404-CML-495, on:

1. the synthesis of fluorescent compounds to determine which of these will react rapidly with small amounts of protein and/or nucleic acids.

2. the preparation, purification and characterization of oxidation-reduction indicator dyes, such as the tetrazolium salts, so that their applicability with respect to the detection of aerosolized bacteria can be studied.

Earlier work by the author indicated that fluorescent compounds can be used to detect organic compounds below the microgram range (1×10^{-6} g.)(1,2). Therefore, it seemed desirable to investigate compounds which on the basis of their structure are expected to show an intense fluorescence. Such compounds may be used to attach reactive groups which can react with proteins and/or nucleic acids and give either an increase, shift or quenching of their fluorescent intensity.

Also earlier work by the author at Synthetical Laboratories, Chicago, Illinois, and at Brooklyn College (after 1950) has been concerned with the synthesis and

properties of tetrazolium compounds and their application to viability of seeds, detection of malignancy in neoplastic tissues, reducing enzymes and in general, reducing functional groups of organic compounds. However, no systematic and accurate work, has been done on the redox potentials of these types of compounds which would help one to predict the structures that would give extreme sensitivity of detecting viability or living organisms.

B Specific Objectives of the Investigation.

1. To synthesize and study the properties of phthalein type of compounds

2. To synthesize and study the properties of the sulfonphthalein type of compounds

3. To synthesize and study the properties of the fluorescein type of compounds

4. To synthesize and study the properties of tetrazolium salt type of compounds and their correesponding formazane

SECTION II

SELECTION OF EXPERIMENTAL METHODS

A. Preparation of Phthaleins, Sulfonphthaleins and Fluoresceins.

The general method employed throughout is to condense aromatic anhydrides of dicarboxylic acids, as for example, phthalic anhydride with an aromatic phenol. Phthalic anhydride, chloro-, and bromo-substituted phthalic anhydrides, o-sulfobenzoic acid anhydride, and halogen-substituted o-sulfobenzoic acid anhydrides were used. A large variety of monocyclic phenols, with various substituents were used as discussed in Sections III and IV.

The condensation procedure was to heat 1 mole of the anhydride with slightly more than 2 moles of the phenol both in the presence and the absence of condensing agents such as concentrated sulfuric acid and anhydrous zinc chloride. It was found that the latter gave better results than heating without a catalyst or sulfuric acid. Typical preparations are described in Sections III and IV.

The melt from the condensation was boiled with water so as to disintegrate the mass and remove the catalyst, then dissolved in 5% sodium hydroxide and the phthalein, sulfonphthalein or fluorescein precipitated by the addition of dilute hydrochloric acid to pH 1.0. The product was then filtered, dried and used for screening directly as

described in Section III, in the screening test for fluorescence. Those compounds which showed appreciable fluorescence with dilution were selected for further study.

B. Method for Rapid Screening for Fluorescence.

The compound was dissolved: (a) in 0.1 N NaOH; (b) chloroform; (c) ethanol so as to give first a concentration of 1 mg/ml. Then it was diluted with the same solvent in order to make solutions containing 1 µg/ml. or less. A sample solution was placed in a 6-inch tube and irradiated from the side with a UV-hand lamp (3660 Å) (3) and the dilution at which fluorescence disappeared noted. From these tests the lower limits, expressed in micrograms/milliter, of the compound at which it gives a noticeable fluorescence was determined.

C. Accurate Method for Determination of Relative Fluorescence.

In the first eight months of the investigation a Farrand Spectrofluorometer was used. In the last 12 months of the investigation an Aminco-Bowman Spectrophotofluorometer was employed. Each instrument has some advantages and also some disadvantages.

The advantages of the Aminco over the Farrand are:

1. Engineering - more compact unit.
2. Lamp - 1000 hours for the blower-cooled Aminco osram lamp while only 100 hours for the Farrand.

3. Slit changing: the Farrand monochromators must be opened to change slits.

4. Wavelength changing: the Farrand has a gear system that has only two speeds and is subject to jamming.

5. Power supply: Battery for the Farrand versus line operated for the Aminco.

6. Controls: either the recorder or the microammeter may be used, but not both together in the Farrand.

7. Cuvet size: the standard size for the Aminco is 10 5 x 10 5 x 46 cm. while the standard size for the Farrand is 10 x 20 x 50 cm.

8. Irradiation: there is a shutter arrangement on the Aminco allowing the sample to be irradiated only when desired.

The advantages of the Farrand instrument are:

1. Filters: a primary filter may be used without any modification.

2. Placement of the lamp: the lamp is far enough removed to prevent any warming of the sample.

The compounds on which accurate measurements of fluorescence were made were fractionated by several methods so as to obtain fractions which had a higher purity than the crude products first obtained by the condensations.

The general method for determining the relative fluorescence of a sample was to prepare a solution in 0.1 N NaOH so as to obtain a solution of 1 mg/ml. then

to dilute successively with 0.1 N NaOH until the resulting solution gave about the same intensity as the blank.

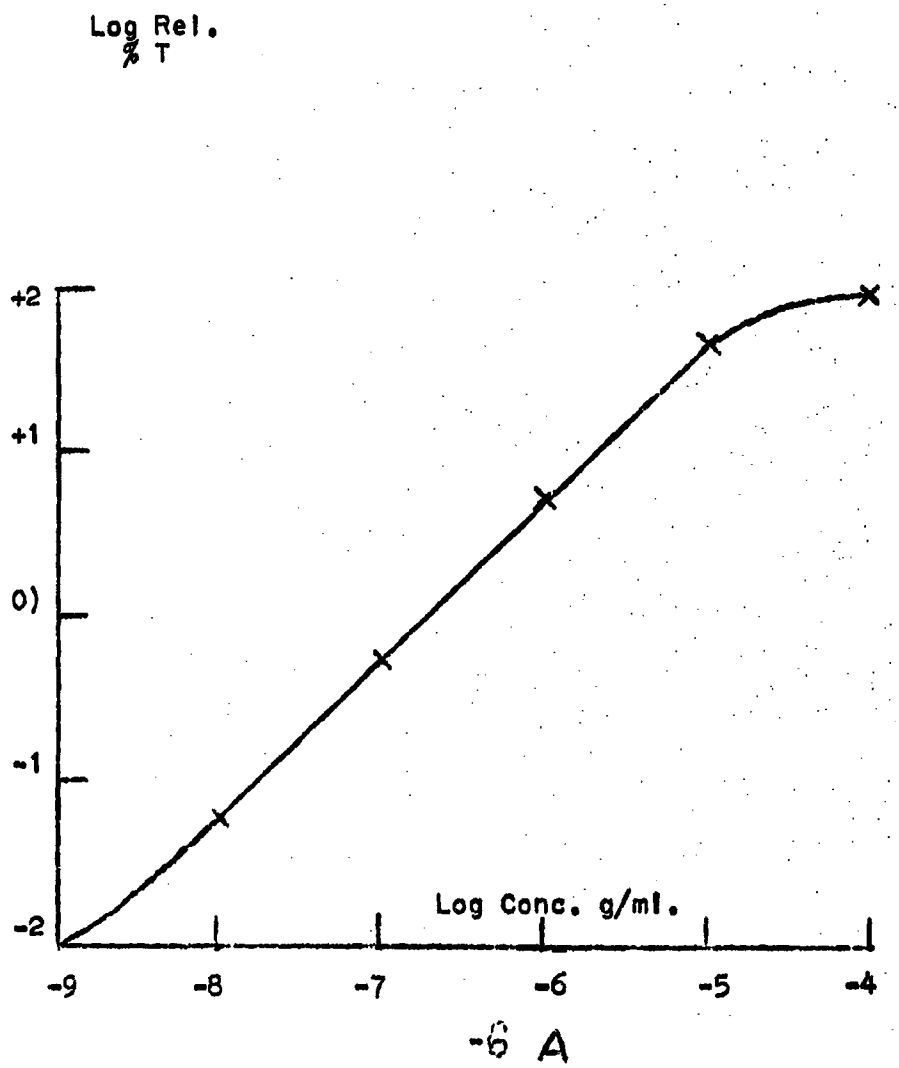
Ordinary distilled water was used at first but the blank was erratic. The procedure adopted was to prepare triple distilled water and use this in preparing the 0.1 N NaOH solution.

The instrument was standardized by using quinine dissolved in 0.1 N H_2SO_4 at 10 µg/ml. and taking serial dilutions to 0.001 µg/ml. The reported (4) activation peak of 350 mµ and fluorescence peak of 450 mµ were obtained.

For fluorescence studies, the peak excitation and fluorescence monochromator settings give values that are read from the photometer in percent transmission. The photomultiplier microphotometer has a meter multiplier attachment that allows different resistors to be placed into the input amplification circuit. The relative percent transmission, therefore, is the product of the meter reading and the meter multiplier; the 1 setting being the least sensitive and the 0.001 being the most sensitive. The relative percent transmission for slit width, photomultiplier tube or pH or solvent must be recorded, since a change in these values will give a different value for the percent transmission. The selection of the photomultiplier tube depends upon the peak fluorescence or emission wavelength, with the 1P28 tube being more sensitive at the lower wavelengths than the 1P21 tube.

Blank solvent values are determined. The plot

Figure 1. Calibration Curve of Log of Relative % Transmission v. Log of Concentration of Quinine in 0.1 N H_2SO_4, 1P21 tube, Slit #3.



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of the log of relative intensity of the sample, minus relative intensity of the blank versus log of concentration should be linear over some range of low concentrations. For example the plot of the log of relative percent transmission versus the log of concentration of quinine is shown in Figure 1.

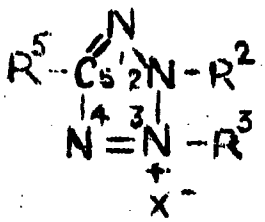
D. General Method for Preparation of Tetrazolium Salts.

A review of the most important methods for the preparation of tetrazolium salts is given in a number of Master's theses (5) of the Graduate School of Brooklyn College, in Nineham's article in Chemical Reviews (6) and in Zdenek's review (7).

The general method followed in the present investigation may be outlined by the following steps:
(a) Preparation of a substituted hydrazone by the reaction of phenylhydrazine or substituted phenylhydrazine and an aldehyde:

$$R^5CHO + R^2NHNH_2 \rightarrow R^5CH=NNHR^2 \qquad (1)$$

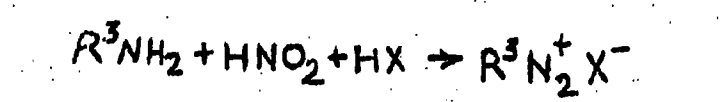
The superscripts R^5 and R^2 are used to denote the positions of the radicals in the tetrazole ring which is ultimately formed as shown in Structure (2).



(2)

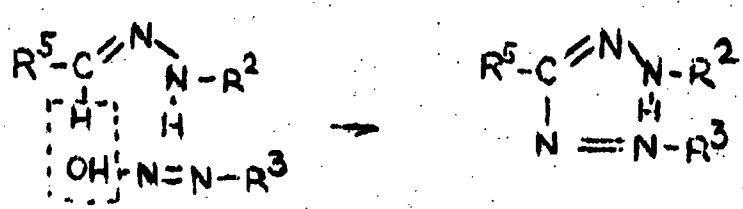
x = halogen

(b) Reaction of the hydrazone with a diazotized amine at alkaline pH: Inspection of Structure (2) indicates that the radical R^5 is derived from the aldehyde reacted with the hydrazine; R^2 is derived from the radical of the hydrazine and R^3 is derived from the diazotized amine which is reacted with the phenylhydrazone to produce the formazan as shown in Structures (3), (4) and (5):



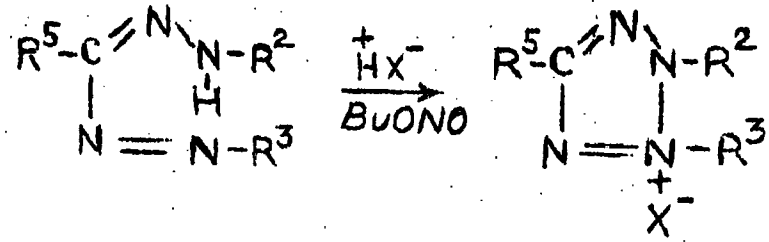
(3)

(4)



(5)

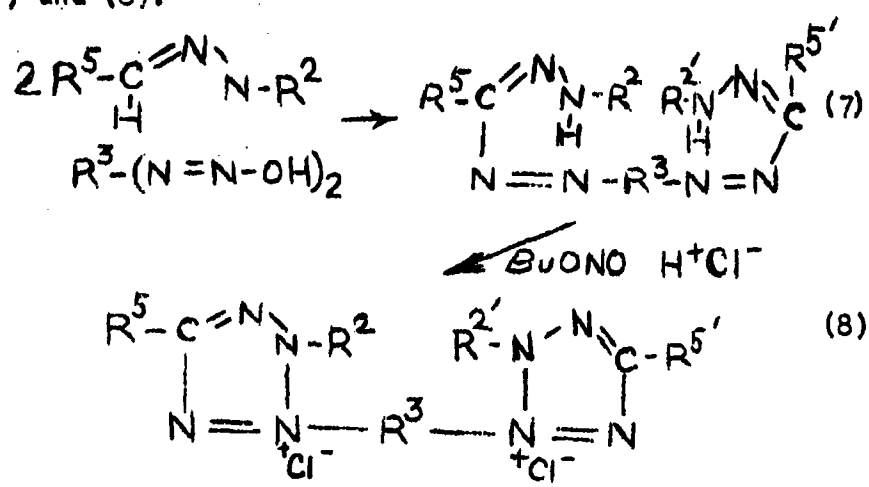
(c) The formazan thus obtained is isolated, purified and then oxidized to close the ring and yield the tetrazolium salt:



(6)

(d) The final step is the isolation and purification of the tetrazolium salt. In practically all cases the salt was the chloride, though in a few instances the bromide was prepared.

For the preparation of the ditetrazolium salts the same general method was employed except one mole of a tetrazotized diamine was used in place of the diazotized amine and 2 moles of the hydrazone as shown in Structures (7) and (8):



(7)

(8)

Typical preparations and discussion of the problems encountered in this phase of the investigation are given in Sections VII and VIII.

SECTION III

PREPARATION OF FLUORESCENT COMPOUNDS AND PRELIMINARY SCREENING

A. Detailed Description of Method for Preparation of Phthaleins and Sulfonphthaleins.

1. Preparation of Anhydrous Zinc Chloride. Zinc chloride was heated until liquid in a metal pot and stirred until viscous. It was then heated for an additional 10 minutes and allowed to cool and solidify; crushed with a pestle until it is a fine powder (while hot), and kept in a dry stoppered vessel, parafinned at the stopper to keep moisture out. The dry powder was weighed rapidly and added to the condensation mixture and the stock bottle resealed.

2. Condensation of Anhydride and Phenol. In an 8-inch test tube, 0.02 moles of the anhydride and 0.04 moles of the phenol were heated until melted, then 4 g. of the anhydrous zinc chloride was added and heated for 4-5 hours in an oil bath at 120-140°C. At this point the melt should be viscous or a solid.

3. Isolation of the Phthalein or Sulfonphthalein. To the melt 10 ml. of water was added and boiled. The mass was poured into 100 ml. of water and mixed with 5 ml. of concentrated hydrochloric acid. If the condensate does not precipitate, it is treated as described below. The precipitate was filtered and washed twice with 25 ml. of dilute hydrochloric acid; the residue filtered and

heated with 15 ml. of 10% sodium hydroxide. 50 ml. of water was added and the solution was allowed to cool. It was then filtered and reextracted with sodium hydroxide until colorless, acidified with hydrochloric acid until the phthalein precipitates, filtered, washed twice with dilute hydrochloric acid and then twice with water, and then dried in an oven at 100°C.

If an oil results on disintegration and acidification of the melt, dissolve in 10% sodium hydroxide by warming to 50°C. Dilute to 500 ml. with water. Warm and stir; filter and acidify filtrate with hydrochloric acid, allow to cool and filter residue. Wash the product with hydrochloric acid and then water and dry in oven at 100°C.

B. Fluorescent Compounds Synthesized.

Table 1 summarizes the substituted phthaleins, sulfonphthaleins, and fluoresceins prepared according to the method described in Section III-A. The first column gives the laboratory number assigned to the preparation (for identification purposes); column 2 gives the anhydride and column 3, the phenol used in the condensation. It will be noted that no attempt has been made to give chemical names to these condensation products, though structures and names have been entered in the notebooks of the project and also in some of the progress reports. The reason is that no attempt was made to isolate a pure compound, determine its constants and establish its identity

except in the case of those compounds which exhibited greater fluorescence intensity than fluorescein. Such compounds are treated in detail in Section IV.

TABLE 1

LIST OF SUBSTITUTED PHTHALEINS, SULFONPHTHALEINS AND FLUORESCEINS PREPARED

Lab. No.	Anhydride (Used in the Condensation)	Phenol
100	o-sulfobenzoic	4,4 bis (4-hydroxyphenol) pentanoic acid (DPA)
101	phthalic	" "
102	tetrahydrophthalic	" "
103	tetrachlorophthalic	" "
104	tetrabromophthalic	" "
105	o-sulfobenzoic	4,4 bis (3,5-dichloro-4-hydroxyphenol) pentamoic acid (tetrachloro DPA)
106	phthalic	" "
108	tetrachlorophthalic	" "
109	tetrabromophthalic	" "
110	o-sulfobenzoic	4,4 bis (3,5-dibromo-4-hydroxyphenol) pentanoic acid
111	phthalic	" "
113	tetrachlorophthalic	" "
114	tetrabromophthalic	" "
115	o-sulfobenzoic	Ethyl 4,4 bis (4-hydroxyphenol) pentanoate (DPA ethylester)
116	phthalic	" "
117	tetrahydrophthalic	" "
118	tetrachlorophthalic	" "

TABLE 1 (continued)

Lab. No.	Anhydride (Used in the Condensation)	Phenol
119	tetrabromophthalic	Ethyl 4,4 bis (4-hydroxylphenol) pentanoate (DPA ethylester)
120	o-sulfobenzoic	4,4 bis (2,6-ditert-butylphenol)
121	phthalic	" "
123	o-sulfobenzoic	2,6-ditert-butyl-α-methoxy-p-cresol
124	phthalic	" "
125	tetrahydrophthalic	" "
127	phthalic	2,6-ditert-butyl-α-dimethylamino-p-cresol
129	o-sulfobenzoic	4,4-methylene bis (β-tert-butyl-o-cresol)
130	phthalic	" "
131	tetrahydrophthalic	" "
133	tetrabromophthalic	4,4-methylene bis (6 tert-butylphenol)
138	tetrabromophthalic	resorcinol

C. Preliminary Screening of the Compounds Synthesized.

1. Method. The general method employed for rapid screening of the fluorescence was to the lower limits of fluorescence upon dilution, using the general method outlined in Section II-B. The initial solution in 0.1 N NaOH containing 1 mg/ml. was diluted with water by a factor of ten until a solution was obtained which upon irradiation with the hand UV-lamp failed to show any fluorescence. The

solution which showed barely noticeable fluorescence was noted as the lower limit and expressed either as mg/ml. or µg/ml.

2. Results of Screening. Table 2 gives a summary of screening tests of the 20 compounds synthesized:

TABLE 2

SUMMARY OF SCREENING FOR FLUORESCENCE OF SUBSTITUTED PHTHALEINS, SULFONPHTHALEINS AND FLUORESCEINS

Compound Lab. No.[a]	Solvent	Result[b]	Lower Limit[c]	pH of Final Dilution
100	.1N NaOH	+	0.03 µg/ml	4.69
	$CHCl_3$	-		
	C_2H_5OH	+		
101	.1N NaOH	+	0.6 µg/ml	10.78
	$CHCl_3$	+		
	C_2H_5OH	+		
102	.1N NaOH	+	1.0 mg/ml	11.1
	$CHCl_3$	+		
	C_2H_5OH	+		
103	.1N NaOH	+	0.01 µg/ml	6.6
	$CHCl_3$			
	C_2H_5OH	+		
104	.1N NaOH	+	0.96 µg/ml	7.2
	$CHCl_3$	+		
	C_2H_5OH	+		
105	.1N NaOH	+	0.75 µg/ml	11.1
	$CHCl_3$	+		
	C_2H_5OH	+		
106	.1N NaOH	+	0.5 µg/ml	11.40
	$CHCl_3$	+		
	C_2H_5OH	+		
108	.1N NaOH	+	0.01 µg/ml	10.00
	$CHCl_3$	-		
	C_2H_5OH	-		
109	.1N NaOH	+	0.25 µg/ml	11.25
	$CHCl_3$	+		
	C_2H_5OH	-		
110	.1N NaOH	+	1.5 µg/ml	11.40
	$CHCl_3$	+		
	C_2H_5OH	+		
111	.1N NaOH	+	3.0 µg/ml	11.45
	$CHCl_3$	+		
	C_2H_5OH	+		

TABLE 2 (continued)

Compound Lab. No.[a]	Solvent	Result[b]	Lower Limit[c]	pH of Final Dilution
113	.1N NaOH	+	3.0 µg/ml	11.50
	$CHCl_3$	+		
	C_2H_5OH	+		
114	.1N NaOH	+	1.5 µg/ml	11.35
	$CHCl_3$	+		
	C_2H_5OH	-		
115	.1N NaOH	+	0.09 µg/ml	9.10
	$CHCl_3$	+		
	C_2H_5OH	+		
116	.1N NaOH	+	0.18 µg/ml	10.20
	$CHCl_3$	+		
	C_2H_5OH	+		
117	.1N NaOH	+	0.09 µg/ml	8.51
	$CHCl_3$	+		
	C_2H_5OH	+		
118	.1N NaOH	+	0.09 µg/ml	9.20
	$CHCl_3$	+		
	C_2H_5OH	+		
119	.1N NaOH	+	0.75 µg/ml	10.40
	$CHCl_3$	-		
	C_2H_5OH	+		
120	.1N NaOH	+	1.5 µg/ml	11.50
	$CHCl_3$	-		
	C_2H_5OH	-		
121	.1N NaOH	+	1.5 µg/ml	11.45
	$CHCl_3$	-		
	C_2H_5OH	+		
123	.1N NaOH	+	0.37 µg/ml	11.40
	$CHCl_3$	+		
	C_2H_5OH	+		
124	.1N NaOH	+	0.18 µg/ml	10.10
	$CHCl_3$	+		
	C_2H_5OH	+		
125	.1N NaOH	+	0.3 µg/ml	9.15
	$CHCl_3$	+		
	C_2H_5OH	+		
127	.1N NaOH	+	---	---
	$CHCl_3$	-		
	C_2H_5OH	-		
129	.1N NaOH	+	5.0 µg/ml	11.35
	$CHCl_3$	+		
	C_2H_5OH	+		
130	.1N NaOH	+	1.5 µg/ml	11.60
	$CHCl_3$	+		
	C_2H_5OH	+		
131	.1N NaOH	+	20.0 µg/ml	11.41
	$CHCl_3$	+		
	C_2H_5OH	+		

TABLE 2 (continued)

Compound Lab. No.[a]	Solvent	Result[b]	Lower Limit[c]	pH of Final Dilution
133	.1N NaOH	+	10.0 µg/ml	11.45
	$CHCl_3$	+		
	C_2H_5OH	+		
138	.1N NaOH	+	0.005 µg/ml	5.80
	$CHCl_3$	+		
	C_2H_5OH			

[a] For nature of compound, see number in Table 1.
[b] + = fluorescence, - = no fluorescence, at a concentration of 1 mg/ml.
[c] The concentration below which fluorescence fades.

D. Tentative Conclusion from Screening Tests for Fluorescence.

In attempting to evaluate the data summarized in Table 2, it should be noted that though the products were not purified and their structure not determined that it is possible to discern certain trends. Though the pH of the solution changed from about 12.45 to that noted in column 5 of Table 2 (which in most cases is in the alkaline range) one may make the reservation that some compounds might fluoresce more strongly at some particular pH's than the one shown in column 5 of Table 2. It should also be noted that it may be more useful to test each product at various pH ranges in order to determine the optimum pH for fluorescence. However, even with this admittedly limited type of test, certain trends are clearly discernible which are summarized in the following paragraphs:

1. Generally, fluorescence in aqueous alkali is greater

than in chloroform or ethanol. A number of compounds do not fluoresce in chloroform, but do so in alcohol. It will be noted that the pH of the diluted solutions at the lower limits varies from 6-11. This was due to the fact that pH drops as the solution in 0.1 N NaOH is diluted. The solution of compound 138, after dilution, was the same as distilled water: 5.8.

2. Fluorescence decreases a thousand fold or more by partial hydrogenation of the phthalic anhydride structure (compare 101 and 102).

3. Compounds 100, 101, 103, 104, 105, 106, 109, 110, 111, 113, and 114 are related since general structure is the same but the substituents vary.

4. Generally sulfonphthaleins are more fluorescent than phthaleins (compare 100 and 101).

5. Compounds 103 and 104 indicate that chlorine on the phthalic structure has a greater effect than bromine.

6. The tentative conclusion is that chlorine has a greater effect than bromine. Compounds 108 and 109 both contain 8 halogens but compound 108 fluoresces about 25 times more than 109.

7. Compounds 109 and 113 are tetrachloro and tetrabromo; compound 109 exhibits about 10 times greater fluorescence than 113. This effect will be further studied.

8. In compounds 110 and 111 again we find that sulfonphthaleins have twice the activity of phthaleins.

9. The effect of 8 chlorine atoms and 8 bromine atoms is

shown by the compounds 108 and 114. The former compound is about 15 times more effective than the latter.

10. The effects of sulfonphthalein is shown by compounds 100, 115, and 116. Esterification decreases fluorescence. Compounds 102 and 117 are compared. These effects are also shown in compounds 119 and 120.

11. Compounds 115 and 120 are compared. Both are sulfonphthaleins and both have carboxylic ester groups but in 120 we have introduced in the phenol 4 tertiary butyl groups and the fluorescence is decreased by 15 times.

12. Comparing 122, 123, 124, 125, 127, 129, 130, 131, and 133, the striking fact emerges that the introduction of an α-dimethylamino group (127) decreases fluorescence by a factor of 1000. The rest of the trends are mainly that the sulfonphthaleins are more fluorescent than phthaleins and the introduction of terbutyl groups do not enhance fluorescence.

13. The most fluorescent compounds are numbers 103, 108, and 138. The last one is a simple fluorescein structure with 4 bromine atoms on the phthalic anhydride molecule. Numbers 103 and 108 represent tetrachloro and octachloro phthaleins. It appears reasonable to expect tetrachloro and octachlorofluorescenn and tetrachloro and octachloro sulfonfluoresceins to be more active.

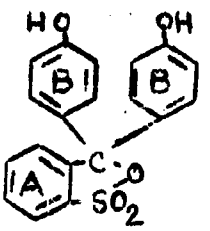
14. The general conclusion is that a substituted fluorescinn exhibits greater fluorescence than the substituted phthaleins and sulfonphthaleins.

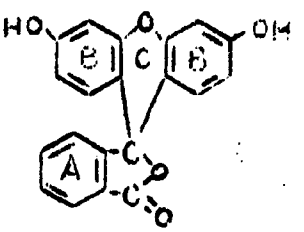
SECTION IV

SYNTHESIS OF COMPOUNDS HAVING GREATER FLUORESCENT INTENSITY THAN FLUORESCEIN

A. Theoretical Considerations.

Inspection of the structure of a typical sulfonphthalein (Structure 9) and a typical fluorescein (Structure 10) shows that the main difference between the two structures





Structure 9

Structure 10

is that the two phenolic rings (B) combine by an oxygen bridge to form another six-membered ring designated by (C) in Structure 10. It was assumed therefore, that the introduction of this new six-membered structure permits a greater extended resonance and hence a greater intensity in fluorescence. It was further assumed that if this assumption was correct, further extension of resonance could be introduced:

By introducing polycyclic structure at the region (A) of the molecule (Structure 10), by using a dicyclic or a

tricyclic acid anhydride or at the region (B) of the molecule by using a dicyclic or a tricyclic phenol. Therefore, a number of preliminary runs were made using first naphthalic (1,8) anhydride with resorcinol and later quinoline anhydride, and then various naphthalene diols.

B. Synthesis of Complex Fluoresceins.

Table 3 summarizes the condensation products obtained by reaction of polycyclic acid anhydrides with resorcinol and also with polycyclic phenols. Table 4 gives a summary of a few exploratory runs to determine whether fluorescent products could be obtained by reaction of o, and m-phenylene diamines and acid anhydrides in accordance with the following postulated equation for m-phenylene diamine:

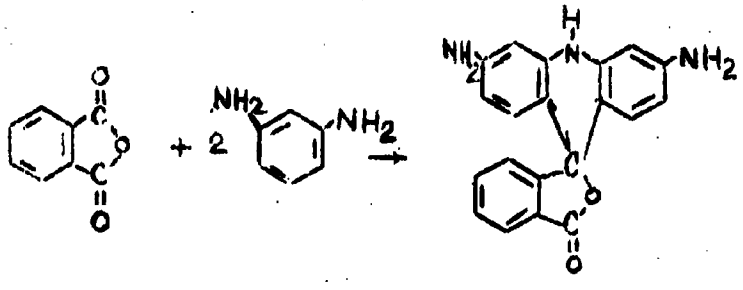


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TABLE 3

SUMMARY OF COMPLEX FLUORESCEINS PREPARED BY REACTION OF POLYCYCLIC [redacted] ANHYDRIDES AND MONO AND DICYCLIC PHENOLIC COMPOUNDS

Lab. No.	Anhydride (Used in the condensation)	Phenol	Remarks
230	1,8-Naphthalic	Resorcinol	0.0008 μg/ml[a]
231	1,8-Naphthalic	1,3-Naphthalenediol	Poor fluorescence
232	1,8-Naphthalic	1,4-Naphthalenediol	Poor fluorescence
233	1,8-Naphthalic	1,5-Naphthalenediol	Poor fluorescence
234	1,8-Naphthalic	1-Hydroxyanthraquinone	Poor fluorescence
235	1,8-Naphthalic	1,8-Dihydroxyanthraquinone	Poor fluorescence
236	1,8-Naphthalic	8-Quinolinol	Poor fluorescence
237	1,8-Naphthalic	4-(m-Nitrophenylazo) resorcinol	Poor fluorescence

[a]Lower limit of fluorescence detection.

TABLE 4

SUMMARY OF CONDENSATION PRODUCTS BETWEEN o, AND m-PHENYLENEDIAMINES AND 1,8-NAPHTHALIC ANHYDRIDE

Lab. No.	Diamine	M.P.	A.M.[a]	F.M.[b]	Lower Limit
258	m-phenylene[c]	350°C+	370 500	420 525	10^{-14} g/ml. 10^{-14} g/ml.
259	o-phenylene[d]	350°C+	350	395	10^{-8} g/ml.

[a] Activation monochromator
[b] Fluorescence monochromator
[c] In 0.1N NaOH
[d] Poor solubility in NaOH so 10 mg. first dissolved in 15 ml. of 6N HCl and brought to 1 liter with 0.1N NaOH.

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C. Screening of Complex Fluoresceins.

The method of screening was the same as described in Section III-C. Of the compounds listed in Table 3, naphthol fluorescein and also the condensate of phthalic anhydride and brominated resorcinol showed the most promising fluorescence in that the lower limits of detection were far lower than that of fluorescein. In fact, even the crude naphthol fluorescein showed lower limits of detection below 1 x 10^{-12} g/ml. Therefore, a systematic study was undertaken to obtain a pure naphthol fluorescein and determine its properties.

Other interesting compounds were synthesized at a later date from β-resorcylic acid and anhydrides. The main fluorescent fractions are shown in Table 5.

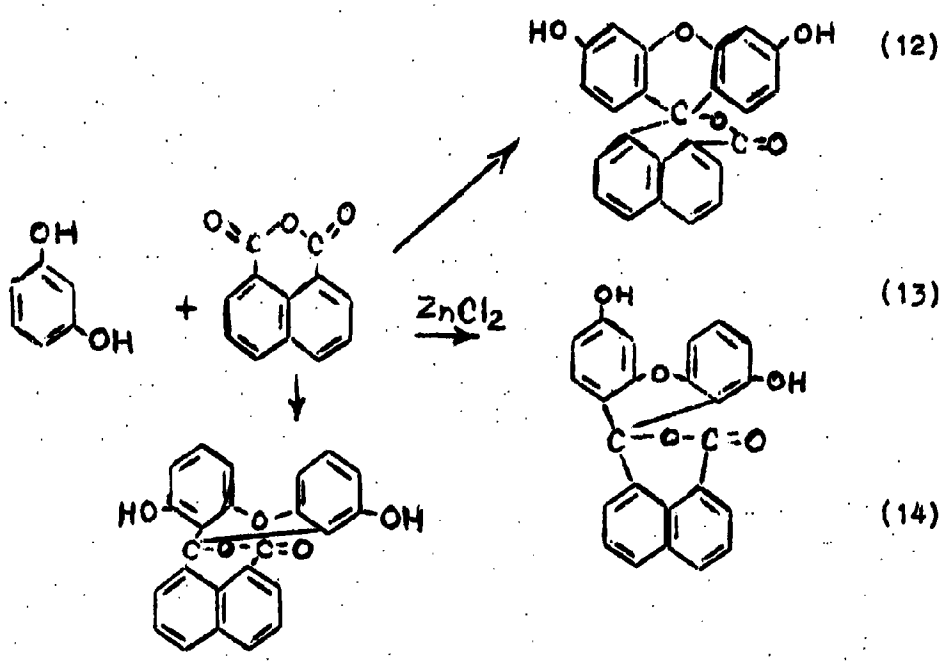
TABLE 5

SUMMARY OF CONDENSATION PRODUCTS BETWEEN β-RESORCYLIC ACID AND NAPHTHALIC AND PHTHALIC ANHYDRIDES

Lab. No.	Anhydride	M.P.	A.M.	F.M.	Lower Limit
253	Phthalic	319-320°C	290,320, 380,475 (370)	525 (475)	10^{-19} g/ml.
254	1,8 Naphthalic	198-201°C	290,380 475 (380)	525 (420)	no data

D. Preparation and Purification of Naphthol fluorescein.

1. Nature of the possible products in the condensation of naphthalic anhydride and resorcinol. There are a number of possible structures in the condensation of naphthalic anhydride and resorcinol as shown by the following formulas:



That the naphtholfluorescein prepared according to the method described in the following subsection consists of several products, each having different fluorescence, was shown by simple fractionation with paper chromatography as described in this section. Therefore, a large amount of work has been done on the problems involved in the fractionation of the reaction products of the condensation of naphthalic anhydride and resorcinol.

2. Preparation of naphtholfluorescein. Recently an old reference to the synthesis of naphtholfluorescein was found (8) and this method does not differ markedly from the conditions that have been described here. Using three 8" test tubes as reaction vessels, a total of 24 grams (.062 moles) of 1,8-naphthalic anhydride and 27 grams (.25 moles) of resorcinol were heated to 190-200°C. After the addition of $ZnCl_2$, the reaction vessels were heated for 4 hours. The crude yield was 16.5 grams. This was fractionated by dissolving in 5% NaOH at room temperature, filtered and treated with 6N acetic acid until a product precipitated (A - brown). After one hour, the mixture was filtered and 6N acetic acid added to give precipitate B (orange-brown). The filtrate was evaporated under vacuum to yield precipitate C (red-orange). Approximately equal quantities of A and B were obtained and only small quantities of C. Fluorescent studies are given in Table 7.

Another method of naphtholfluorescein synthesis, using the acid chloride of 1,8-naphthalic anhydride, has been reported (9).

3. Chromatographic Separation of the components of crude fluorescein. A solution of 10 µl. representing 5 µg. of fluorescein was chromatographed on Whatman #1 paper, using ascending technique with 3% NaCl, 5% phenol and 0.1N aqueous NH_3 (1:1:1) as the developing solvent.

The spots obtained has the following characteristics:

Rf

Spot A .20 - red and nonfluorescent

B .62 - yellow and fluorescent

C .90 - dim blue and fluorescent

The material was fractionated in the same manner as naphtholfluorescein and the fractions A and C are shown in Table 6 with C possibly being the yellow form of β fluorescein (10) and A being ϒ fluorescein (11).

TABLE 6

FLUORESCENT DATA ON VARIOUS FLUORESCEIN FRACTIONATION PRODUCTS

	A.M.	F.M.	Lower Limit	M.P.
fluorescein A	290,320 380,475	515	10^{-13} g/ml.	r to br at 250°C no m.p. at 350°C+
fluorescein C	same	same	10^{-18} g/ml.	y to r at 210°C m.p. 305-7°C

4. Fractionation of crude naphtholfluorescein. This product is described in Section D-2. The physical constants are given in Table 7. A plot of the log of concentration vs. log of relative percent transmission is shown in Figure 2, for the peak activation (500 mμ) at the peak fluorescence (525 mμ). The expected Beer's Law curve is seen from 10^{-6} g/ml. to 10^{-10} g/ml. Below this concentration, there is detectable but erratic fluorescence (see also Section IV).

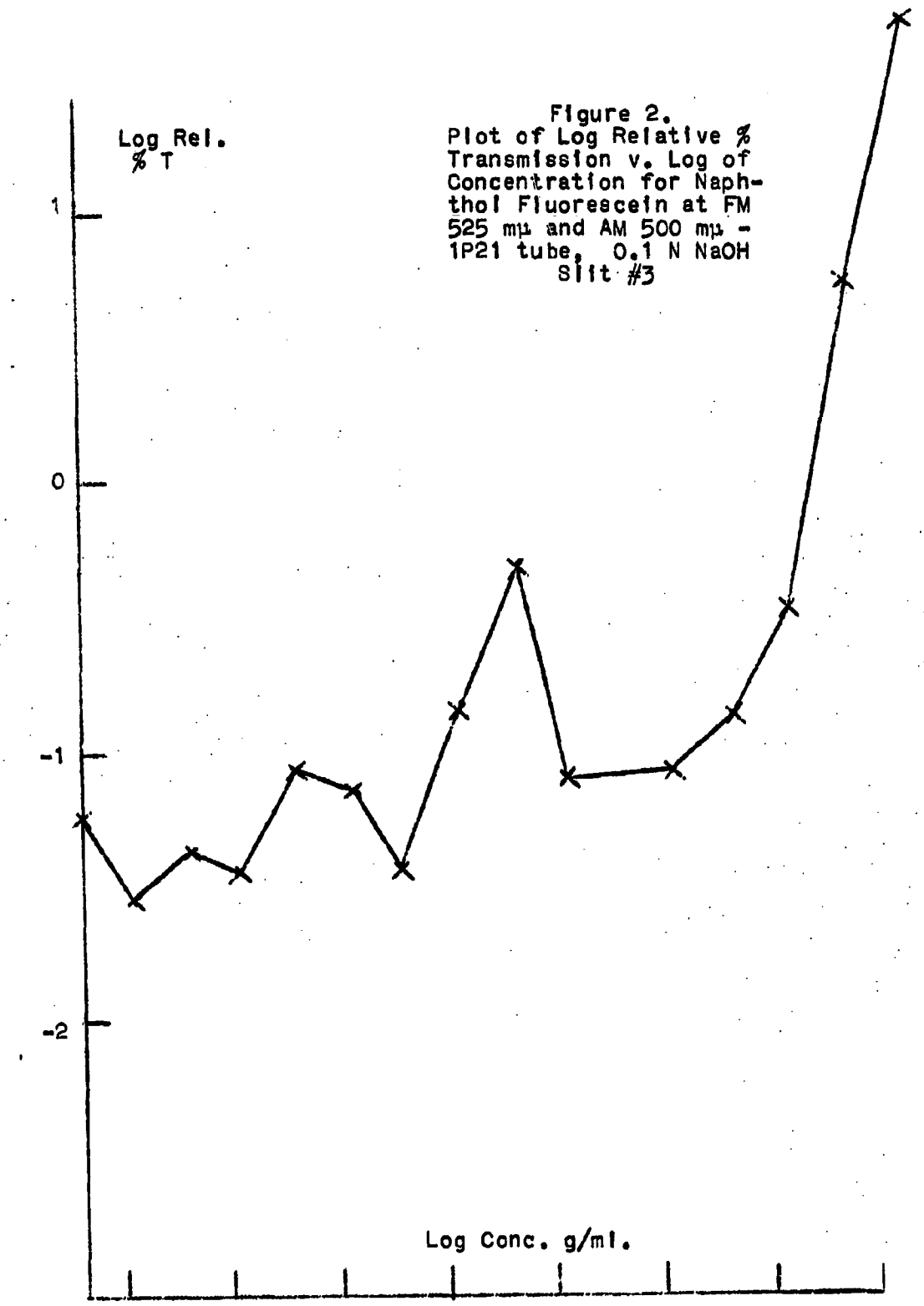


Figure 2.
Plot of Log Relative % Transmission v. Log of Concentration for Naphthol Fluorescein at FM 525 mμ and AM 500 mμ - 1P21 tube, 0.1 N NaOH Slit #3

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TABLE 7

PHYSICAL CONSTANTS OF NAPHTHOLFLUORESCEIN FRACTIONS

	A.M.	F.M.	Lower Limit[a]	Rf.	I.R.	M.P.
A	290,320,380 475,500	525	10^{-13} g/ml.	.74	2.7-3μ slight	350°C+ - no melting
B	same	525	10^{-21} g/ml.[b]	.62	2.7-3μ strong	225-28°C
C	same	525		.61	--	350°C+ - no melting

[a] 0.1N NaOH as a solvent.
[b] In 0.001N NaOH, only 10^{-12} g/ml.

Carbon, hydrogen and oxygen analysis was performed on a naphtholfluorescein crude that was purified by precipitation from a methanol-ether solution by addition of water. This product was probably a mixture (m.p. 293°C) but the data indicates that the assumed formula is correct or nearly correct.

$C_{24}H_{14}O_5$ molecular weight 382.4

	% C	% H	% O	Total
calculated	75.35	3.68	20.97	100.00
found	72.26	3.99	20.1	96.35

5. Purification of naphtholfluorescein by acetylation and benzoylation followed by hydrolysis. It is expected that naphtholfluorescein having several free hydroxyl groups would react with acetic anhydride and with benzoyl chloride

and yield the corresponding acetyl and benzoyl derivatives, these can be purified by crystallization until fractions with constant physical properties are obtained, then hydrolyzed to pure naphtholfluorescein.

(a) Acetylation of naphtholfluorescein. Naphtholfluorescein was refluxed for 4 hours with acetic anhydride and pyridine. The cooled reaction mixture was poured into ice water and filtered (m.p. of 108-13°C. for the dried product). It has the same fluorescence maxima as naphtholfluorescein, but lacks one of the activation peaks (320 mμ) and has a lower limit of 10^{-15} g/ml.

Hydrolysis of the acetylated compound was attempted by heating at reflux for one hour with 10% NaOH. The cooled reaction mixture was precipitated with 6N HCl and the product was filtered and dried (m.p. 178-84°C.). This melting point is similar (191°C.) to that obtained by A. Terrisse (8) for the mono acetylated naphthol fluorescein.

I.R. data on the hydrolyzed compound indicates strong absorption at 2.7-2.9 (OH peak).

(b) Benzoylation of naphtholfluorescein. Naphtholfluorescein (1 gm.) was dissolved in 10 ml. of 10% NaOH and 0.8 gm. of benzoyl chloride was added. The contents of the tube were shaken for 20 minutes and then allowed to cool. An oily mass resulted that was separated from the liquid layer and dissolved in 5% NaOH and filtered. The filtrate (I) was precipitated with dilute acetic acid, filtered again to remove the precipitate A and the filtrate (II)

was treated with acetic acid until a second precipitate B resulted. This orange precipitate was filtered and dried. The filtrate (III) was again treated with acetic acid and the third precipitate C was obtained. In 0.1N NaOH only B showed appreciable fluorescence when irradiated with a 3660 Å lamp. All three compounds are phosphorescent. B was assumed to be the benzoylated compound, on the basis of I.R. data, with a melting point of 121-122°C. and a lower limit of fluorescence detection of 10^{-15} g/ml. Rf values were determined for this compound using 5% phenol, 3% NaCl and 1% NH_3 in aqueous solution as the developing solvent: Rf = .73. This compound was redissolved in 5% NaOH and re-precipitated with acetic acid. No spot could be located on paper chromatography with this compound. The original .73 Rf spot was assumed to be naphtholfluorescein.

Attemped hydrolysis of benzoylated naphtholfluorescein using 10% NaOH and heating for one-and-a-half hours gave a product with the same physical properties indicating no hydrolysis. 6N HCl gave no indicated hydrolysis products.

It is believed that benzoylation of naphtholfluorescein takes place at both phenolic sites and at the carboxylate ion. This was checked by benzoylation of naphthalic anhydride which gave a product; this test was negative for attempted acetylation.

There is an I.R. peak that is found for the benzoylated product but not for the acetylated. Benzoylation of the acetylated product does, however, show this peak.

SECTION V.

USE OF COMPOUNDS WITH LARGE FLUORESCENCE INTENSITY FOR DETECTION OF ORGANIC COMPOUNDS BY TAGGING

A. Theoretical Considerations.

One of the chief objectives of the present investigation is the preparation of a fluorescent compound which will react rapidly with small quantities of protein and/or nucleic acids. The fluorescein molecule itself is not expected to react with proteins or with nucleic acid. However, it offers good possibilities as a basic structure on which to attach functional groups known to be reactive with proteins and/or with nucleic acids. Among the functional groups which have been considered to be introduced into the basic structures, the following have been tentatively assigned priority for investigation:

(a) Introduction of sulfonic acid groups

(b) Introduction of a nitro group which then could be converted to an amino group by reduction

(c) Conversion ofthe amino to a diazonium group stabilized by a fluoroborate ion to form a relatively stable salt

(d) Conversion of the amino group to dimethylamino

(e) Conversion of the amino group to an isocyanate and particularly to an isothiocyanate (12) function

(f) Conversion of the sulfonic acid to the N-dimethylsulfonamide - $SO_2N(CH_3)_2$

Some progress has been made in this direction as described in the following pages.

B. Sulfonation of Naphtholfluorescein.

1. Exploratory runs in the sulfonation of naphtholfluorescein. (a) Naphtholfluorescein (2 gms.) was heated for 2 hours at 60°C. with 12 ml. of 20% fuming H_2SO_4, poured into cold water and precipitated as the calcium salt. This solution was evaporated to dryness and the product gave a positive test for sulphur by sodium fusion techniques. Paper chromatography indicated a number of products. There was no success in attempted isolation of these products.

(b) The above procedure was modified using 6 ml. of 20% fuming H_2SO_4 and the product was precipitated as the barium salt. A positive test was given for sulphur and two spots were obtained by paper chromatography. Analysis for sulphur indicates 2.54%. A formula of $C_{48}H_{26}O_{16}BaS_2$ would have 6.05% sulphur. This compound on this basis is obviously contaminated.

(c) The same procedure as (b) except that the temperature was 80-100°C. Analysis for sulphur indicates 4.96%. Purple crystals were obtained in (b) and (c).

(d) The sodium salt was precipitated after a reaction at 120-140°C. for three hours with 20% fuming H_2SO_4. A red compound resulted that gave a positive test for sulphur.

2. Projected Experimentation. In process is sulfonation using the most fluorescent naphtholfluorescein which will be isolated as its sodium salt.

C. Nitration of Naphthalfluorescein.

Two different methods of nitration were used. One analogus to phthalic anhydride nitration (13) yielded a product with a melting point of 222-225°C. The second method was a nitration of acenaphthenequinone and oxidation to give 4-nitro naphthalic anhydride. Literature values for this melting point are 220°C (14) and 230°C. (15). The obtained melting point of 211-214° indicates that this product might be contaminated with the 3 nitro isomer (16). I.R. spectra for both synthesis products were similar.

Attempted synthesis of nitro naphtholfluorescein from resorcinol and nitro naphthalic anhydride gave a compound that did not melt at over 350°C. This will be purified for further synthesis. Also the characterization of both nitro starting materials is in progress.

SECTION VI.

PROBLEMS ENCOUNTERED IN THE PREPARATION, PURIFICATION AND DETERMINATION OF ACTIVITY OF FLUORESCENT COMPOUNDS

Only a brief summary can be given on this topic, since any extensive discussion would involve considerable space. However, for the purpose of this report the following problems may be listed with some remarks as to whether the problem has been completely solved.

1. The selection of the proper catalyst for the condensation of both phthaleins, sulfonphthalein, and fluorescein-type of compound: Though we have used $ZnCl_2$ exclus-

ively, there is no doubt that the condensing agent gives rise to other products which are difficultto remove. Condensation without a catalyst, even with greater heating time, has failed to give any appreciable yield of condensate.

2. It is quite certain on the basis of available data that at least two isomeric naphtholfluoresceins are formed. One fraction shows stronger -OH absorption bands in the infrared spectra, than the other. This is believed to have both hydroxyl groups free as shown in Structure 10, Section IV-A. More work is required for the final characterization of each isomer.

3. A more basic approach is needed to the correlation of chemical structure and fluorescence. Work has been started in this direction by Lt. Miller at Fort Detrick Laboratories.

4. A better method for the introduction of sulfonic acid groups into fluorescent compounds is needed and work is in progress towards that direction.

5. Finally, the measurements of fluorescence at extremely low concentrations (1×10^{-12} to 1×10^{-18}) and even below must be carefully scrutinized. For example, if we assume a molecular weight of 382 for naphtholfluorescein, then if we get a signal at a concentration of 1×10^{-18} g/ml, we are dealing with about 1560 molecules and if we get a signal at 1×10^{-21}, the number of molecules is only 1.56 --- a very unlikely behavior. The

results at low concentrations are very erratic.

The possibility that molecules are trapped in pockets in the glass and are not transferred in dilutions must be considered. Experimentation using broken glass in low concentration fluorescent solutions to absorb fluorescent molecules indicates that this is a factor. This absorption experimentation and the attempted coating of glass with silicones is in progress. Proper mixing is a very important factor at low concentration and this might be part of the absorption factor.

Glassware should be cleaned in hot nitric acid and rinsed with distilled water and then blank solvent. Dichromate cleaning solution and soap solutions should be avoided.

SECTION VII.

PREPARATION OF TETRAZOLIUM SALTS

A. Plan for the Preparation of Formazans.

The general method for the preparation of formazans was outlined in Section II-D. In the present section a list of all the formazans which have been planned will be given and typical preparations will be described, as well as the difficulties encountered in the isolation, purification and characterization of the product.

Table 8 gives a summary of the monoformazans and the corresponding tetrazolium salts that have been scheduled for preparation. Compounds Laboratory number 533 to 553

inclusive were suggested by Lt. Miller of Fort Detrick Laboratories. Table 9 lists the diformazans and the corresponding salts that have been scheduled for preparation. One plus (+) before the number of the compound indicates that the formazan has been prepared and purified. Two pluses (++) indicates that the formazan has been converted to the tetrazolium salt and the compound isolated and submitted to Fort Detrick Laboratories for further study.

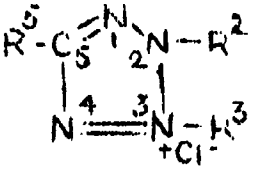
(15) 

TABLE 8

MONOFORMAZANS AND TETRAZOLIUM SALTS LISTED FOR PREPARATION [a]

Lab. No.	Radicals in Positions Indicated R5	R2	R3
++500	Phenyl	Phenyl	o-Tolyl
++501	p-Anisyl	Phenyl	o-Tolyl
++502	Methyl	Phenyl	Phenyl
++503	Phenyl	Phenyl	p-Tolyl
++504	Phenyl	Phenyl	Phenyl
++505	Piperonyl	Phenyl	o-Anisyl
++506	Phenyl	Phenyl	p-Anisyl
++507	Phenyl	Phenyl	α-Naphthyl
508	Piperonyl	Phenyl	Phenyl
+509	Piperonyl	Phenyl	o-Tolyl
510	Piperonyl	Phenyl	p-Tolyl
+511	Phenyl	Phenyl	m-Tolyl
+512	Phenyl	Phenyl	o-Anisyl

TABLE 8 (continued)

Lab. No.	Radicals in Positions Indicated R^5	R^2	R^3
+513	Phenyl	Phenyl	β-Naphthyl
+514	Piperonyl	Phenyl	m-Tolyl
+516	Piperonyl	Phenyl	p-Anisyl
517	Piperonyl	Phenyl	α-Naphthyl
+520	p-Anisyl	Phenyl	p-Anisyl
+521	p-Anisyl	Phenyl	α-Naphthyl
+522	p-Anisyl	Phenyl	Phenyl
+523	Phenyl	p-Nitrophenyl	Phenyl
+524	m-Nitrophenyl	Phenyl	Phenyl
+525	Phenyl	Phenyl	p-Nitrophenyl
527	n-Propyl	Phenyl	Phenyl
528	Piperonyl	p-Nitrophenyl	Phenyl
529	Piperonyl	p-Nitrophenyl	p-Anisyl
530	Piperonyl	p-Nitrophenyl	p-Nitrophenyl
531	p-Anisyl	p-Nitrophenyl	p-Anisyl
532	p-Anisyl	p-Nitrophenyl	p-Nitrophenyl
533	p-Nitrophenyl	Phenyl	Phenyl
534	p-Nitrophenyl	Phenyl	Phenyl
535	Phenyl	2,4-Dinitrophenyl	Phenyl
536	p-Nitrophenyl	2,4-Dinitrophenyl	Phenyl
537	p-Nitrophenyl	p-Nitrophenyl	Phenyl
538	p-Anisyl	Phenyl	Phenyl
539	m-Anisyl	Phenyl	Phenyl
540	Phenyl	p-Nitrophenyl	p-Nitrophenyl

TABLE 8 (continued)

Lab. No.	Radicals in Positions Indicated R5	R2	R3
541	α-Naphthyl	Phenyl	Phenyl
542	9-Anthrnayl	Phenyl	Phenyl
543	Ethylene	Phenyl	Phenyl
544	o-Aminophenyl	Phenyl	Phenyl
545	m-Aminophenyl	Phenyl	Phenyl
546	p-Aminophenyl	Phenyl	Phenyl
547	Phenyl	Methyl	Phenyl
549	p-Hydroxyphenyl	Phanyl	Phenyl
550	p-Chlorophenyl	Phenyl	Phenyl
551	p-Fluorophenyl	Phenyl	Phenyl
552	p-Carboxyphenyl	Phenyl	Phenyl
553	p-Thiophenyl	Phenyl	Phenyl
++554	Phenyl	p-Nitrophenyl	p-Iodophenyl

[a] + = Formazan prepared and purified; ++ = Tetrazolium salt prepared and purified.

555 Phenyl Phenyl Biphenyl

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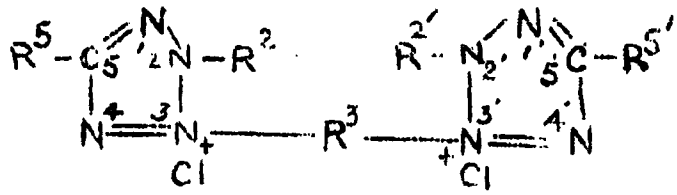
TABLE 9

DIFORMAZANS AND DITETRAZOLIUM SALTS
LISTED FOR PREPARATION



(16)

Lab. No.	Trivial Name	R^2, 2'	R^5, 5'	R^3
++703	Regular Tetrazolium Blue	Phenyl	Phenyl	o-Dimethoxy-biphenylene
++705	p-Anisyl Blue	Phenyl	p-Anisyl	o-Dimethoxy-biphenylene
++706	Piperonyl Blue	Phenyl	Piperonyl	o-Dimethoxy-biphenylene
++707	Veratryl Blue	Phenyl	Veratryl	o-Dimethoxy-biphenylene
++708	m-Nitroneotetrazolium chloride	Phenyl	m-Nitrophenyl	Biphenylene
++709	Neotetrazolium chloride	Phenyl	Phenyl	Biphenylene
++710	p-Nitro-blue tetrazolium chloride	p-Nitrophenyl	Phenyl	o-Dimethoxy-biphenylene

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B. Description of the Preparation of some Monoformazans and Monotetrazolium Salts.

1. Preparation of 2,3,5-triphenyl tetrazolium chloride (lab. no. 504). (a) Preparation of the formazan. 94 ml. of aniline, 125 ml. of water and 210 ml. of concentrated HCl were chilled and added to 100 gm. of ice. This was diazotized with a solution of 70 gm. of $NaNO_2$ dissolved in 150 ml. of water. The diazotized material was added to a solution of NaOH and benzalphenyl hydrazone through a dropping funnel at 0-10°C. over a period of 2 hours. The stirring was continued for an additional half-hour, cooled for 24 hours and filtered. The NaOH solution was prepared by dissolving 250 g. NaOH in 250 ml. of water, cooling and adding 700 ml. of methanol. This was added to 200 gm. of the hydrazone in 4 liters of methanol.

(b) Purification of the formazan. The formazan was washed with 100 mls. of methanol and suspended in a solution of 250 ml. of methanol and 250 ml. of acetone. After boiling for 5 minutes, it was cooled and filtered and resuspended in 2 liters of boiling water for 10 minutes. At 60°C. it was filtered and washed with 50 ml. of boiling methanol. The product was dried in a vacuum desiccator.

(c) Oxidation of the formazan to the tetrazolium salt. 50 gm. of the formazan was suspended in 250 ml. of ethanol and 250 ml. of $CHCl_3$. To this solution 32 ml. of butyl nitrite was added and dry HCl gas was bubbled through the chilled (5°C.) solution until it was saturated. The oxi-

dation is usually complete in 2 hours and the red mixture is then colorless. The bubbling was discontinued, but stirring continued for 1 hour. Charcoal was added and the mixture was allowed to stand overnight.

(d) Isolation and purification of the tetrazolium salt. The solvents were stripped off under vacuum to leave approximately 20 ml. Methanol (30 ml.) was added. The solution was boiled with charcoal, filtered and 300 ml. of dry ether and 25 ml. of acetone were added while the walls of the flask were scratched until crystals formed. After 1 hour, the crystals were filtered and recrystallized to give a constant melting point after drying in a vacuum desiccator.

2. Preparation of 2,5-diphenyl-3-o-tolyl tetrazolium chloride (lab. no. 500). The same procedure was followed as described in the previous section (B-1) except -toluidine was diazotized.

3. Preparation of 2-p-nitro phenyl-3-p-iodophenyl-5-phenyl tetrazolium chloride (no. 554). (a) 30 gm. of p-nitrophenyl hydrazone was suspended in 1.1 liters of ethanol and a solution of KOH (prepared from 35 gm. of base dissolved in 40 ml. of water which was cooled and mixed with 150 ml. of ethanol) was added with stirring. p-iodoaniline solution (prepared by dissolving 25 gm. in 30 ml. of warm water, then adding 35 ml. of concentrated HCl, cooling to 0°C. and diazotizing with 10 gm. of $NaNO_2$ in 20 ml. of water) was added over a period of 1 hour with stirring and maintenance of 0°C. temperature. The stir-

ring was continued for 1 hour and then the solution was allowed to remain in the cold overnight.

(b) The formazan was filtered and washed with 50 ml. of methanol and suspended in a mixture of 75 ml. of methanol and 75 ml. of acetone which was then boiled, allowed to cool and filtered twice. The solid was suspended in 1 liter of boiling water for 5 minutes, cooled to 60°C., filtered, washed and dried.

(c) 15 gm. of the iodo formazan in 350 ml. of ethanol and 350 ml. of $CHCl_3$ with 15 ml. of butyl nitrite was oxidized as in section B-1-(c).

(d). The procedure was the same as section B-1-(d) except that the tetrazolium compound is precipitated by adding the alcoholic solution to 500 ml. of dry ether. The product was filtered and dried; two recrystallizations gave a constant melting product.

4. Preparation of p-anisyl blue. (a) 125 gm. of p-anisaldehyde was dissolved in 500 ml. of methanol and heated to boiling. To this a phenyl hydrazine solution (120 ml. of the hydrazine and 250 ml. of methanol) was added while stirring. Yield was 200 gm. 100 gms. of the hydrazone was dissolved in 600 ml. of pyridine and cooled to -5°C. The slow addition of the fast blue salt was performed, stirring over a period of 5 hours and then stirring for an additional hour cooled overnight and precipitated with 400 mls. of methanol. The remainder of the procedure (b,c,d) was the same as described in section B-1.

5. Preparation of tetrazolium blue. (a) 100 gm. of benzalphenyl hydrazone was dissolved in 1.5 liters of pyridine. To this hydrazone, 440 gm. of fast blue salt was added over a period of 6 hours while the temperature was kept at 0-5°C. The remainder of the procedure (b,c,d) was the same as described in section B-1.

6. Preparation of p-nitro tetrazolium blue. (a) 100 gm. of benzal-p-nitrophenylhydrazone was dissolved in 1.2 liters of pyridine, then cooled to -5°C. 380 gm. of blue salt was added to the above solution under very vigorous stirring over a period of 4-6 hours. After all the blue salt was added, the stirring was continued for an additional hour. The formazan was allowed to stand in the cold for 24 hours.

(b) The same procedure was followed as described in section B-1-(b).

(c) 20 gm. of p-nitro-blue formazan was suspended in 320 ml. of dioxane and 300 ml. of tetrahydrofuran and 32 ml. of butyl nitrite, cooled to -10°C. Then, while stirring, dry HCl was bubbled into it until a supersaturated solution was obtained. It requires about 6 hours for the completion of oxidation. 8 ml. more of butyl nitiite was added with charcoal and allowed to stand for 24 hours.

(d) The product was filtered and the solvent stripped to 30 ml. Methanol (150 ml.) and charcoal were added and filtered. The filtrate was slowly added with vigorous stirring to a 3 liter wide-mouth flask containing 1.5 liters of

dry ether. The product was filtered, dried and recrystallized to a constant melting point.

(NOTE: The "fast blue" mentioned in Sections B-4 (a) and 5 (a) refers to stabilized o-dianisidine tetrazonium salt obtained from Dyestuff Division of Koppers Co.)

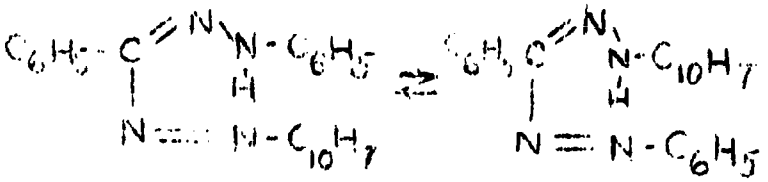
SECTION VIII.

PHYSICAL CONSTANTS AND CHARACTERIZATIONS OF FORMAZANS AND TETRAZOLIUM SALTS AND PROBLEMS ENCOUNTERED

A. Criteria of Purity.

One of the most vexing problems in this phase of the work has been to establish criteria for the purity of (a) the formazans and (b) the tetrazolium salts.

1. Monoformazans. The problems encountered with the monoformazans are not very great though tautomerism is possible between the 2- and 3- positions. For example, if one starts with: benzalphenylhydrazone and reacts it with diazotized α-naphthylamine, the formazan is identical to that formed when one starts with benzal-α-naphthylhydrazone and reacts it with diazotized aniline. It is assumed that the hydrogen bond between positions 2- and 3-, as shown in structure (17) permit tautomerism:



(17)

In general, the criteria of purity as agreed upon in this project for the monoformazans are:

(1) No change in melting point after two successive crystallizations.

(2) No change in I.R. spectra, maximum U.V. absorption valuee and Rf values after two successive crystallizations.

The use of melting point with monoformazans as rapid criteria of purity in purification by crystallization is feasible, since generally the melting points are below 200°C. and do not decompose appreciably near the melting point, though after melting, the crystallized melt obtained by cooling does not give the same melting point again.

However, there are some inherent difficulties in the purification of the monoformazans, particularly if the radicals on positions 2, 3 and 5 have nitro groups or halogens. The purification depends on the extent of the reaction between the hydrazone and the diazotized amine which probably at the alkaline pH of the reacting medium is the diazotate ion. The solvent and temperature, and perhaps, pH, have an effect. This is shown by the preparation of the formazan of 2-p-nitrophenyl-3-p-iodophenyl-5-phenyl formazan. The formazan was prepared by three different methods: in the first, the usual aqueous-alcohol system with the addition of aqueous potassium hydroxide was used; in the second, the hydrazone was dissolved in pyridine and the diazotized p-iodoaniline was added; in the third, the hydrazone was dissolved in dioxane and the diazotized p-iodoaniline was added. Only the first method gave a formazan which on purification yielded a product melting at 184-185°C which is listed in the literature.

2. Diformazans. The purification of diformazans is inherently more difficult since besides the desirable reaction of one mole of tetrazotized diamine with 2 moles of the hydrazone, a monoformazan results at the same time. This has been extensively investigated by Seligman et al (17) in connection with the preparation of p-nitrotetrazolium blue formazan or 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy)-4,4-biphenylene diformazan. Seligman's group first reported that they obtained a mixture of monoformazan melting at 210°C. and diformazan melting at 257°C. They reported a separation with boiling dioxane in which the monoformazan was insoluble and the diformazan soluble. In a later paper they changed their directions to continuous extraction for 7 days with boiling benzene. It has not been possible to confirm the findings of the Seligman group. The pure diformazan has been obtained by a completely different method as described in the preceding section and the melting point of the compound is the same as stated by Seligman et al. 256-257°C.

3. Tetrazolium Salts. The criteria of purity for the tetrazolium salts are even more confused due to the following: (a) the melting points of the tetrazolium salts are decomposition points and are affected by the rate of heating; (b) tetrazolium salts solvate, tend to separate as oils, and not infrequently crystallize with various numbers of molecules of the solvent of crystallization. Numerous examples can be cited that by dissolving portions of the same lot in methanol,

or in isopropyl alcohol and then precipitating with ether, products are obtained with different melting points; (c) finally, there is the possibility of polymorphism and possible stereoisomerism in some of the tetrazolium salts.

All of the above considerations led to a detailed and exhaustive study of the oxidation of formazans under conditions which will yield products which can be isolated readily, purified and given uniform constants.

It has been found that the following factors play an important role during the oxidation of the formazan and closing of the ring: (a) solvent system; (b) temperature; (c) presence of water as for example, addition of concentrated aqueous hydrochloric acid gives inferior results to the use of anhydrous hydrogen chloride; (d) evaporation of the solvent after oxidation under reduced pressure instead of evaporation in contact with air; (e) method of crystallization. The last point is illustrated by the purification of p-nitrobluetetrazolium chloride. The same crude was used. By varying the method of crystallization, one lot gave crystals melting at 212°C., another at 184-185°C. and the third, crystals that did not melt at 300°C. The lots melting at 184-185° and 212°C. gave substantially identical I.R. spectra. However, there still remains the suspicion that the two lots may behave differently towards enzyme systems. These are problems which have to be investigated.

B. Physical Constants of Formazans and Tetrazolium Salts.

This phase of the work is still in progress. The data given in Table 10 are tentative. A large amount of data are missing because it is considered a waste of time to determine constants unless the purity and nature of the compound has been established. Work on the Rf values of both formazans and tetrazolium salts is just beginning.

TABLE 10

MELTING POINTS AND INFRARED SPECTRA OF FORMAZANS AND TETRAZOLIUM SALTS

Lab. No.	No. Cr.[a]	M. P.	I.R.[b]
500 F[c]	2	125-8	-
501 F	2	101-3	-
503 F	2	144-6	-
504 F	2	159-61	-
506 F	3	148-51	-
507 F	2	157-8	-
509 F	3	142-4	-
511 F	2	124-6	-
512 F	2	146-8	-
513 F	3	154-6	
514 F	3	160-2	-
515 F	2	101-2	-
516 F	3	159-61	-
519 F	2	118-9	-
520 F	2	116-8	-
521 F	2	120-1	-

TABLE 10 (continued)

Lab. No.	No. Cr.	M.P.(°C)	I.R.
522 F	2	107-8	-
523 F	2	162-4	-
524 F	2	114-6	-
525 F	2	147-50	-
554 F	2	184-5	-
554 S[d]	2	194-5	-
703 S	3	252	+
705 S	2	197	+
706 S	2	206	+
707 S	2	199	+
708 S	2	240	+
709 S	...	133-7 255-6	+
710 S	...	184-5 212-13 over 300	+

[a] No. Cr. = number of crystallizations of product for which the value is reported.
[b] I.R. = infrared spectra; + = determined; - = not determined.
[c] F = formazan.
[d] S = tetrazolium salt.

C. The Use of Tetrazolium Salts for Detection of Radiation and also for Protection Against Radiation.

A complete bibliography of all reported literature on tetrazolium salts is contemplated. However, attention should be called to three papers by Gierlach and Krebs (18) from the Army Medical Research Laboratory at Fort Knox, Ky. between

1949-1952. Copies of these papers are being submitted to the Physical Defense Division of Fort Detrick Laboratories. The present author has not seen any further reports on investigations of this angle. [redacted] Therefore, it is suggested that some exploratory work should be done with the pure tetrazolium salts furnished to the Fort Detrick Laboratories to determine their possible use in radiation studies.

SECTION IX

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